Recessive Dystrophic Epidermolysis Bullosa Phenotype Is Preserved in Xenografts Using SCID Mice: Development of an Experimental In Vivo Model

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Recessive dystrophic epidermolysis bullosa (RDEB) is a subgroup of hereditary blistering diseases characterized by repetitive wounding and healing with subsequent extensive scarring. The purpose of this study was to establish a xenograft model that retains the RDEB phenotype and thus might be used as an experimental in vivo model to explore the molecular and biochemical mechanisms of the chronically wounded phenotype of RDEB. Full-thickness, tumor-free RDEB skin tissues were grafted onto the dorsum of severe combined immunodeficiency (SCID) mice. At 4, 8, 12, and 24 weeks after grafting, the xenografts were removed for examination. Immunofluorescence studies were performed using species-specific antibodies to human class I antigen, mouse class I antigen, human type IV and VII collagens and with cross-reacting antibody against bullous pemphigoid antigen (BPA). Staining with the antibody to human class I antigen, W6/32, and with the antibody to mouse class I antigen, 20.8.4s, confirmed the species-specific results obtained with the type IV and type VII collagen and laminin antibodies. The RDEB grafts showed essentially no staining with the type VII collagen antibody. Antibodies against laminin and BPA showed normal staining patterns in RDEB grafts. There was an overall paucity of anchoring fibrils in the grafts when examined with electron microscopy. Blisters could be induced in these grafts with minor trauma and showed a sublamina densa separation by immunomapping and electron microscopy. As late as 24 weeks post-transplantation, the RDEB grafts remain human, are not significantly replaced by mouse cells, and retain the RDEB disease phenotype. *J Invest Dermatol 98:191–197, 1992*

Hereditary epidermolysis bullosa (EB) is a group of genetic disorders of the skin characterized by marked fragility and a tendency to form blisters and erosions in response to minor trauma. There are more than 12 hereditary types of EB, which are subclassified into four major forms: dominant EB simplex, recessive junctional EB, dominant dystrophic EB, and recessive dystrophic EB [1–3].

All dystrophic forms of EB feature repetitive wounding and healing of the skin associated with severe scarring. A special feature of RDEB patients is a tendency to develop aggressive squamous cell carcinomas in areas of severe scarring. These cancers lead to high morbidity and mortality at a relatively early age [4].

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Abbreviations:
AF: anchoring fibrils
BPA: bullous pemphigoid antigen
DLAM: Department of Laboratory Animal Medicine
EB: epidermolysis bullosa
EM: electron microscopy
IF: immunofluorescence
LM: light microscopy
PBS: phosphate-buffered saline
RDEB: recessive dystrophic epidermolysis bullosa
SCID: severe combined immunodeficiency

The pathogenesis of RDEB remains unclear; however, two hypotheses have been extensively studied. One hypothesis is that the RDEB skin has a rudimentary or diminished number of anchoring fibrils (AF), resulting in skin fragility [5]. Tidman and Eady demonstrated that there is an absence or severely diminished number of AF in both clinically blistered and non-blistered skin areas. The other hypothesis is that there is an increased production of skin collagenase [6,7]. Skin collagenase has been shown to be increased in both clinically blistered and non-blistered skin areas. Increased production of collagenase might lead to enhanced breakdown of AF, which are predominantly composed of type VII collagen [8,9], thus resulting in blisters.

There are few well-established in vivo models of RDEB that can be used to enhance our current understanding of this disease. Recently, Bruckner-Tuderman et al reported a spontaneous sheep model of RDEB that has similar clinical features and lacks type VII collagen and well-formed AF [10]. An alternative to spontaneous animal models is a xenograft system employing human RDEB tissues that could be used for in vivo experiments. Various investigators have reported prolonged maintenance of normal [11] and diseased human skin such as psoriasis [12,13], lamellar ichthyosis [14], epidermolytic hyperkeratosis [15], and malignant melanoma [16] on athymic mice.

The purpose of this study was to establish an experimental in vivo model of RDEB using the xenograft system that might be used to explore the mechanisms of pathogenesis and carcinogenesis of this chronically wounded phenotype. We used a xenograft system of transplanting RDEB grafts onto severe combined immunodeficiency (SCID) mice. To establish a valid in vivo model, it was extremely important to demonstrate that the xenografts remain human and that they retain the disease phenotype.

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MATERIALS AND METHODS

SCID Mice SCID mice 4–6 weeks of age were purchased from the Department of Radiation Oncology at Stanford University and housed in the research animal facility of the Department of Laboratory Animal Medicine (DLAM) at Stanford. Each SCID mouse was treated with weekly oral antibiotics, sulfamethoxazole/trimethoprim, as part of a routine procedure for SCID mouse housing in the Stanford DLAM. Mice were used for grafting at 6 weeks of age.

Human Subjects Grafts were obtained from five different RDEB patients and two different healthy subjects. All RDEB patients were examined and diagnosed through the Epidermolysis Bullosa Clinic at Stanford University Hospital and all were entered into the National Epidermolysis Bullosa Registry. All five patients were male; their ages were 34, 24, 18, 26, and 7 years. Clinically, all five patients had skin fragility with an extensive, mutilating form of scarring with contractures of minor and major joints, and an autosomal recessive inheritance pattern. Patient 2 (age 24), patient 3 (age 18), and patient 4 (age 26) have developed multiple squamous cell carcinomas and patient 4 has recently succumbed to metastatic squamous cell carcinoma. The tissue examinations of all five patients for diagnosis revealed non-inflammatory subepidermal blisters by light microscopy, rudimentary or decreased numbers of AF in skin examined ultrastructurally, and a blister cleavage plane beneath the lamina densa when lesional skin is analyzed by immunomapping or with an electron microscope. The RDEB grafts were excised from non-tumorous, non-blistered areas of the trunk, extremities, or foreskin. The two healthy skin grafts were obtained from a healthy, male infant whose foreskin was donated after circumcision and from trunkal skin of a healthy, 32-year-old woman without skin disease.

Grafting Technique

Preparation of Human Grafts: Full-thickness elliptical skin, 2.2–2.3 cm in length and 0.8–0.9 cm in width, was obtained from each of the five RDEB patients and the two healthy subjects using sterile technique. After excision, the subcutaneous fat of RDEB grafts was removed with scissors prior to transplantation. The prepared grafts were kept in sterile normal saline with added penicillin and streptomycin and transplanted onto SCID mice within 2 h after harvesting.

Preparation of Graft Bed: Mice were anesthetized with nembutol (0.07 mg/gm mouse weight) intraperitoneally prior to grafting. After shaving the hair, epidermis and dermis of appropriate shape and size were surgically removed without injury to vessels in the panniculus carnosus. The exposed suprapannicular tissue rich in blood vessels then served as the graft bed (Fig 1).

Transplantation of Human Grafts onto SCID Mice: Each RDEB and healthy control skin graft was transplanted onto the dorsum of a separate SCID mouse. The grafts were sutured in place with 6-0 prolene material, and the wounds were dressed with Polysporin ointment, Adaptic, Telfa, and Coban. The dressing material and sutures were removed 7–10 d after transplantation. The midline, dorsal location of the grafts prevented the mice from scratching the grafted site. To minimize trauma to the grafts further, each mouse was housed separately.

Skin Tissue Analysis At 4, 8, 12, and 24 weeks after transplantation, blisters were induced on RDEB grafts by gently twisting the graft skin with an eraser head of a pencil. Because of the scarcity of RDEB tissue, it was impossible to biopsy all xenografts at four different time points for the various tissue analyses. Rather, each graft was biopsied at two different time points with three grafts at 4 and 12 weeks and two grafts at 8 and 24 weeks after transplantation. Immediately following the blister induction at each time point, histologic sections were obtained for light microscopic, immunofluorescence, and electron microscopic examinations. Similar trauma was also inflicted on healthy control skin xenografts. The tissues were obtained and vertical sections prepared that allowed the mouse-human junctions to be observed with ease (Fig 2).

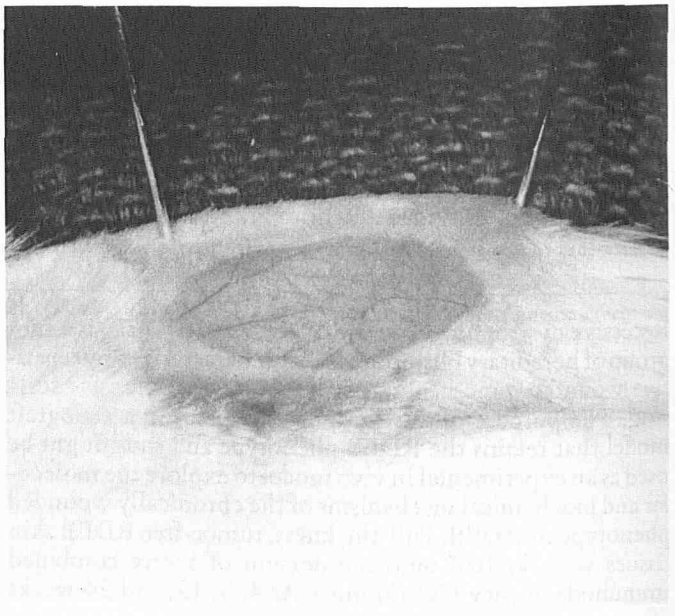


Figure 1. Photograph of the prepared graft bed, panniculus carnosus. After surgical removal of mouse epidermis and dermis, the suprapannicular tissue rich in blood vessels is exposed.

Light Microscopy (LM): Tissues were fixed overnight in 10% formalin and then stained with hematoxylin and eosin using routine methods.

Indirect Immunofluorescence (IF): Mouse monoclonal antibodies, W6/32, directed against human class I antigen [17,18], and 20.8.4s, directed against mouse class I antigen [19], were generously provided by the Department of Medical Microbiology and Immunology (Drs. Peter Parham and Hugh McDevitt) of Stanford University School of Medicine. Optimal IF staining dilutions for each antibody were determined by performing serial dilution experi-

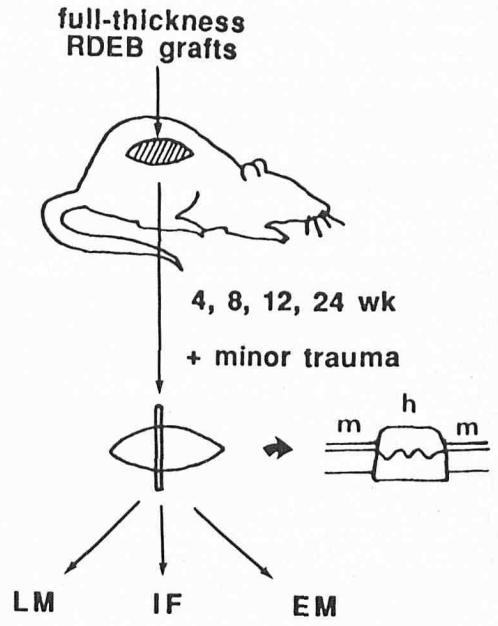


Figure 2. Schematic diagram of grafting and tissue analysis methods. Five full-thickness RDEB grafts were transplanted onto the dorsa of separate SCID mice. At 4, 8, 12, and 24 weeks after transplantation, mild shearing trauma was exerted on the grafts. The vertical sections of tissues were prepared that allowed the mouse (m)-human (h) junctions to be observed easily.

Table I. Tissue Specificities and Working Dilutions of Antibodies

Antibody	Mouse Skin	Human Skin	Working Dilution
α-human class I (W6/32)	−	+	1:20
α-mouse class I (20.8.4s)	+	−	1:80
α-BPA	+	+	1:20
α-laminin	−	+	1:10
α-type IV	−	+	1:50
α-type VII (EBA-2)	−	+	1:20

ments. Normal SCID mouse and human skin were used as control substrates. Murine monoclonal antibodies to human laminin and type IV collagen (lot #2311C3) were kind gifts from Dr. Eva Engvall, La Jolla Cancer Research Foundation, La Jolla, and Dr. John MacDonald, Washington University, St. Louis, respectively. The murine monoclonal antibody against type VII collagen is directed against the carboxy-terminus of the type VII collagen molecule [20]. Serum from a patient with bullous pemphigoid was used to stain BPA. The antibodies used and their tissue specificities and working dilutions are listed in Table I.

Frozen tissue sections were prepared on gelatin-coated slides and were stained with the antibodies on the same day using previously described methods [21]. The primary antibodies were added to the sections and incubated for 45 min in a humidifying chamber. The slides were then rinsed three times for 5 min each in phosphate-buffered saline (PBS) at room temperature. After excess PBS was removed, appropriate fluorescein-conjugated second antibodies were added, and again, the sections were incubated in the humidifying chamber in the dark for 45 min. The slides were rinsed three times for 7 min each in PBS at room temperature. These sections were then mounted with coverslips using polyvinyl alcohol mounting solution and observed with a Zeiss IF microscope equipped with epi-illumination for immunofluorescence. For each primary antibody used, control experiments designed to examine the background staining of the secondary antibody were natural, integral components of the overall IF studies that employed primary antibodies to different antigenic determinants and an identical secondary antibody. As an additional background control, the IF experiments were performed by substituting PBS for the primary antibody.

Electron Microscopy (EM): Tissues were fixed overnight in modified Karnovsky's fixative at 4°C, postfixed in 1% aqueous osmium tetroxide, embedded in epoxy resin (LX 112), and stained with uranyl acetate and lead citrate before examination with Hitachi H300 electron microscope.

RESULTS

Complete graft acceptance was achieved in all seven animals without evidence of either technical failure or immunologic rejection. Figure 3 depicts an RDEB graft 4 weeks after transplantation. In all cases, the human graft was readily identified by gross observation: the paucity of hairs and thicker skin compared with the adjacent mouse skin.

In Fig 4*A*, the junction of human (RDEB xenograft) and mouse skin in the biopsy is designated by the black arrow. To the left of this

Figure 3. Photograph of an RDEB xenograft 4 weeks after transplantation. The human graft was readily identified by the paucity of hairs and thicker skin compared with the adjacent mouse skin.

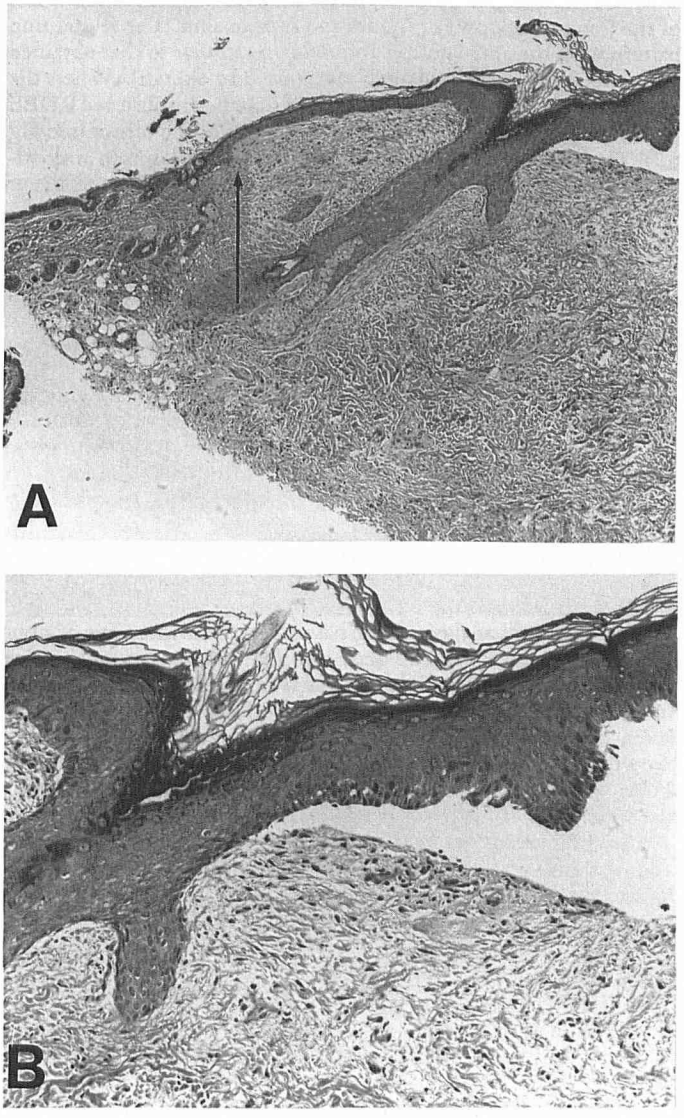


Figure 4. Light micrograph of a blistered RDEB xenograft 8 weeks after transplantation. *A,* The SCID mouse skin, left of the mouse-human junction *(arrow),* is composed of multiple small hairs and thin epidermis, whereas the RDEB skin contains larger human hair follicle, thicker epidermis, and a blister. Magnification ×150. *B,* At higher magnification, the blister cleavage plane is clearly subepidermal. Magnification ×300.

junction is the SCID mouse skin with multiple small hairs and the thin mouse epidermis. On the right of the junction is the RDEB graft with the larger human hair follicle, the thicker epidermis, and a blister. At higher magnification (Fig 4*B*), the cleavage plane of the induced blister is localized to the subepidermal zone.

Staining with the two anti-class I antigen antibodies is shown in Fig 5. Figure 5*A* is the RDEB graft stained with the antibody to human class I antigen, W6/32. The antibody stained the cell surface of keratinocytes in an intercellular pattern reminiscent of pemphigus. The W6/32 antibody also stains fibroblasts, endothelial cells, and cells comprising the adnexal tissues in the dermis. The mouse portion of the biopsy adjacent to the xenograft did not stain with W6/32 human-specific antibody (not shown). Using the antibody to mouse class I antigen, 20.8.4s, there is staining of the mouse portion of the biopsy (Fig 5*B*). Again, there is a similar intercellular (so-called "chickenwire") staining pattern of the cell surface. The RDEB and healthy, control human skin in the biopsies did not stain with mouse-specific 20.8.4s.

Immunolocalization of anti-type IV collagen antibody in the RDEB xenograft is shown in Fig 6*A*. There is a linear staining pattern at the dermal-epidermal junction and around the vessels. The dark, non-staining area of the mouse skin is observed to the left of the junction (arrow) of mouse and human skin. The IF staining pattern with the anti-laminin antibody was similar to that obtained with the anti-type IV collagen antibody (not shown). When the anti-type IV collagen antibody was used to stain the blistered RDEB graft, there was a linear IF pattern at the roof of the blister (see Fig 6*B*). The IF experiments using anti-BPA and anti-laminin antibodies produced similar linear staining patterns at the roof of the blister (not shown).

Because anchoring fibrils are known to be rudimentary or diminished in the skin of patients with RDEB [5], we used an antibody to type VII (anchoring fibril) collagen to determine the expression of this protein in the xenograft (Fig 7). This antibody labeled ungrafted healthy human skin in a linear continuous staining pattern along the dermal-epidermal junction as previously reported [20] (not shown). The antibody to type VII collagen labeled xenografts of healthy skin identically to that of ungrafted healthy skin (Fig 7*A*). In contrast, in Fig 7*B*, the RDEB graft stained at the same dilution showed essentially no staining. A small focus of staining in one graft sampled at 4 weeks after transplantation was the only evidence for any type VII collagen labeling in any of the five grafts. In all other RDEB grafts, type VII collagen staining was completely absent. The results were identical when the antibody was used undiluted.

Ultrastructural analysis of the basement membrane zone of non-blistered areas of the RDEB graft revealed rudimentary or abortive AF that were significantly diminished in number compared with those of the healthy skin xenograft (Fig 8*A,B*). All tissue samplings taken from other RDEB grafts, either blistered or non-blistered, showed a complete absence of well-formed AF. EM analysis of healthy ungrafted skin (not shown) revealed morphology essentially identical to that of the AF observed in healthy skin xenografts. The dermal-epidermal junction of a blistered area RDEB graft was also examined by EM (Fig 8*C*). The cleavage plane of the blister was localized to the sub-lamina densa zone. Ultrastructural evidence of collagenolysis was observed in association with blister formation as demonstrated by the fragmented appearance of the interstitial collagen and of the AF.

A summary of RDEB xenograft tissue analysis is listed in Table II. LM examination revealed a clean, subepidermal blister. The IF results showed that the RDEB grafts stained positively with the antibody to human class I antigen (W6/32) and negatively with the antibody to mouse class I antigen (20.8.4s). With immunomapping, the blister roof stained positively with antisera to BPA, laminin, and type IV collagen. There was a specific lack of staining with type VII collagen antibody, whereas BPA, laminin, and type IV antibodies stained normally. The EM results demonstrated the sub-lamina densa location of the cleavage plane and the rudimentary or diminished number of AF.

DISCUSSION

A hallmark of the RDEB clinical phenotype is that the skin blisters easily in response to minor trauma. Our RDEB grafts blistered with gentle shearing trauma, whereas the healthy normal skin grafts did not blister with the same physical insult. Similarly, we observed a non-inflammatory, subepidermal blister localized to the sub-lamina densa zone and lack of or defective AF, which are key morphologic features of RDEB. The lack of staining with antibody to the carboxy-terminal domain of type VII collagen correlated with the EM observations. However, it is important to emphasize that the RDEB grafts stained normally with BPA, laminin, and type IV collagen antibodies, thus demonstrating that the lack of staining with type VII collagen antibody is disease-specific rather than being due to the transplantation process. The small focus of type VII collagen antibody staining in the one RDEB graft sampled 4 weeks after transplantation (Fig 7*B*) is probably due to residual type VII collagen epitope, although it is formally possible that type VII collagen might have been induced by the grafting process.

The W6/32, 20.8.4s, type IV, and laminin antibody staining results demonstrated the preservation of human characteristics in the RDEB xenografts up to 24 weeks after transplantation. The 20.8.4s antibody was helpful in confirming that the human graft was not replaced with mouse cells during the length of our experiments. We did not observe mouse cells (e.g., keratinocytes, Langerhans cells, or melanocytes) in the epidermis of any of the seven human grafts. This observation is in agreement with that reported by Daynes et al in athymic nude mice [22] where they showed that the human grafts were not infiltrated by host Langerhans cells. Our findings also agree with those of Demarchez et al [23,24], where species-specific antibodies against class I antigen and type IV collagen were used to confirm the preservation of human characteristics in human skin grafted onto athymic nude mice in wound healing observations over a 15-week period after engraftment. Haftek et al also demonstrated a distinct border between mouse and human skin up to 30 d after transplantation by using antiserum against specific keratin polypeptides [25]. The only study that reports invasion of human grafts by host epidermis is that of Graem et al [26]. They reported that 1 of 32 human skin grafts observed over a 48-week period after transplantation showed signs of invasion by murine epidermis (nude mice) when using monoclonal antibodies against blood antigens B and H and OKT6 and by examining the heterochromatin patterns.

There are conflicting data in the literature regarding the fate of the vasculature of the grafted skin. There are reports of survival of endothelial cells in the original vasculature of human grafts [27,28]. However, Dermarchez et al [29] have reported that the original endothelium of human grafts is replaced by mouse endothelial cells and type IV collagen perhaps as early as 2 weeks after transplantation. The antibody 20.8.4s identifies all mouse cells with surface class I antigen, including endothelial cells [19]. However, this antibody did not stain cells within the dermal vessels that were outlined by our type IV collagen antibody, which is human specific. Meanwhile, the antibody to human class I antigen, W6/32, continued to label the dermal endothelial cells of human grafts. Therefore, our results do not demonstrate replacement of the original endothelial cells within the human graft vessels by mouse endothelial cells up to 24 weeks after transplantation.

The reason for the difference in results of the graft endothelium is unclear, although the discrepancy may be in part due to the differences in transplantation methods. We used SCID mouse as the host rather than the Swiss *nu/nu,* athymic mouse used by Demarchez et al [29]. Not only do the immunologic characteristics differ between athymic nude and SCID mice [15,30], but a phenotypic difference may also exist between endothelial cells of the two different species of mice associated with different migration characteristics. Also, we used elliptical, full-thickness grafts, whereas Demarchez et al [29] utilized circular, split-thickness grafts. We have observed that the elliptical grafts heal with less contraction than circular grafts.

There is no reported literature on the nature of other components of the dermis such as fibroblasts and immune cells in human grafts

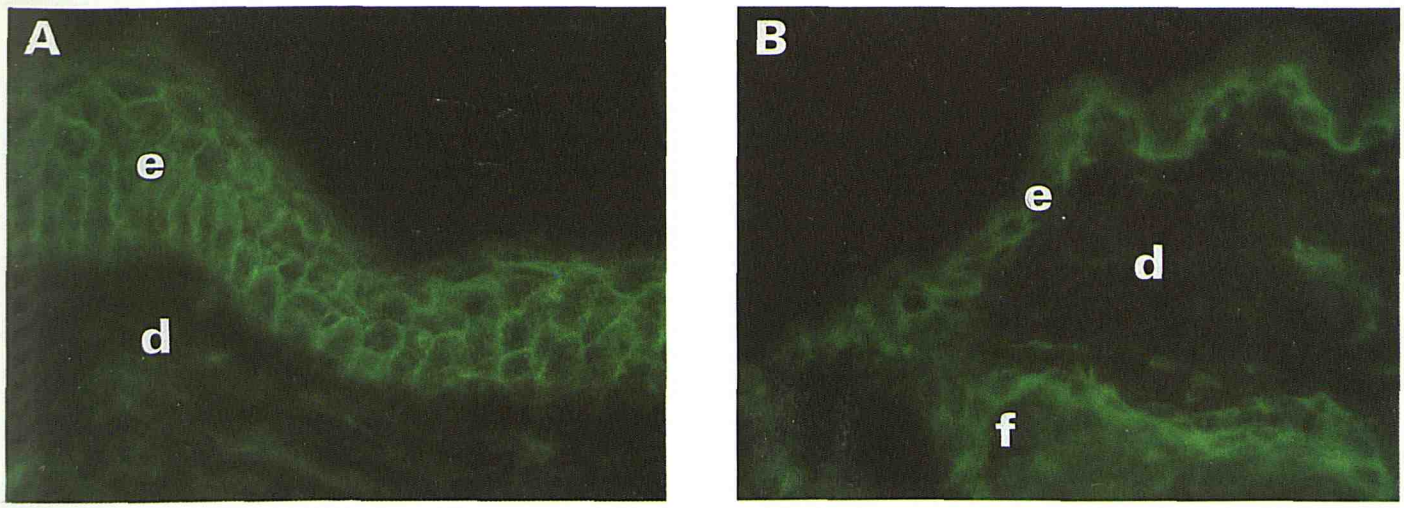


Figure 5. Indirect immunofluorescence staining of RDEB xenograft and adjacent mouse skin with antibodies against class I antigens. Biopsy specimen was obtained 8 weeks after grafting. *A,* Pemphigus-like, intercellular labeling of the human portion (xenograft) of the biopsy by antibody to human class I antigen, W6/32. *B,* Similar intercellular staining pattern of the mouse portion of the biopsy adjacent to the xenograft using antibody to mouse class I antigen, 20.8.4s. *e:* epidermis; *d:* dermis; *f:* hair follicle; *A,B,* Magnification ×400.

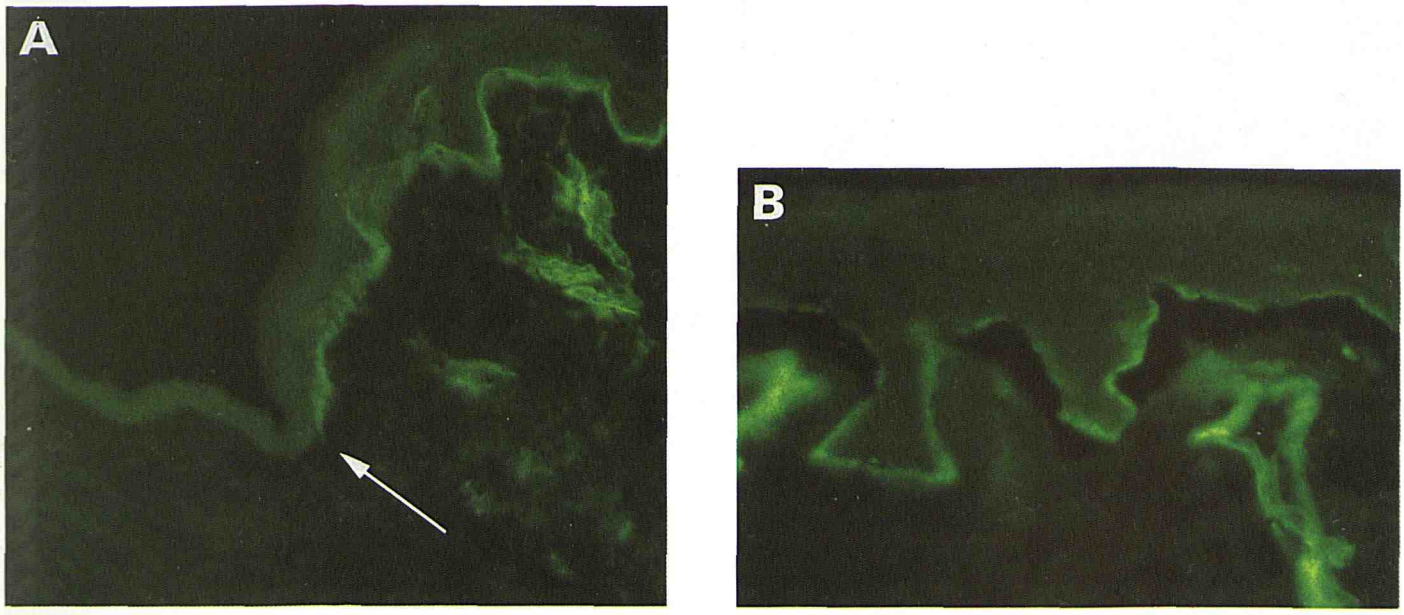


Figure 6. Immunolocalization of human specific, anti-type IV collagen antibody in the RDEB xenografts. *A,* Junctional area of a biopsy specimen obtained 8 weeks after grafting with the RDEB xenograft to the right of the junction *(arrow);* the linear staining pattern of the basement membrane of dermal-epidermal junction and dermal vessels is observed exclusively in the human portion of the biopsy. Magnification ×200. *B,* In blistered areas of the RDEB grafts, the antibody to type IV collagen labels the roof of the blisters. Magnification ×400.

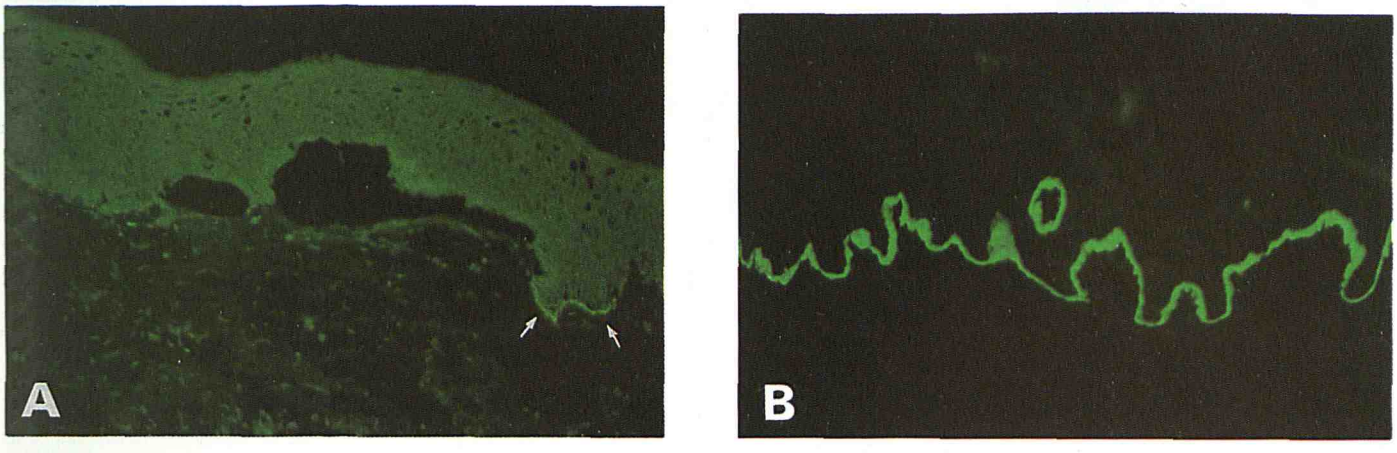
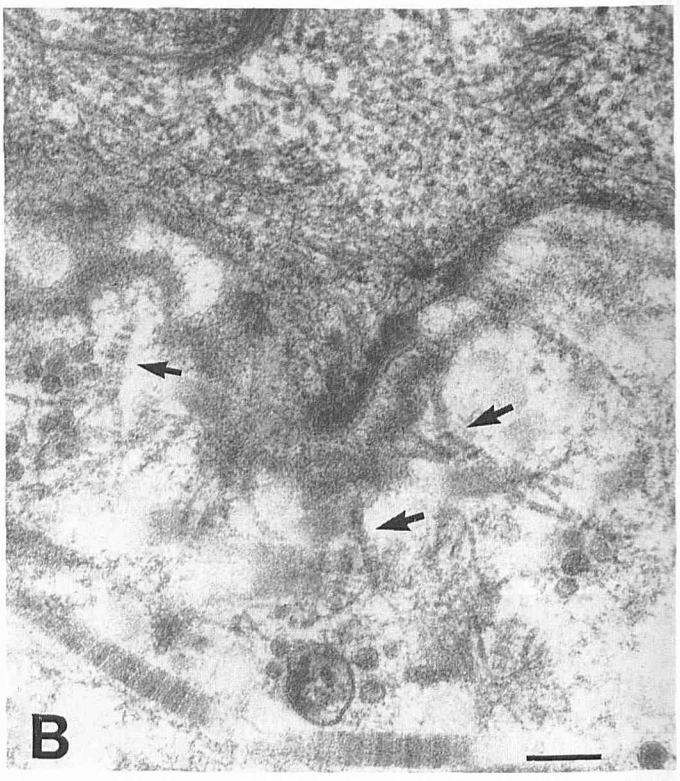


Figure 7. Indirect immunofluorescence labeling with antibody to type VII collagen (carboxy-terminus) of RDEB xenograft *(A)* and xenograft of healthy human skin *(B),* both biopsied 8 weeks after transplantation. *A,* RDEB xenograft showed a significant lack of labeling. Small focus of staining *(arrows)* in this RDEB graft was the only evidence for any type VII collagen labeling in any of the 5 RDEB grafts. *B,* Xenograft of healthy skin stained in a linear continuous pattern along the dermal-epidermal junction, a pattern similar to that of ungrafted healthy skin. *A,B,* Magnification ×200.



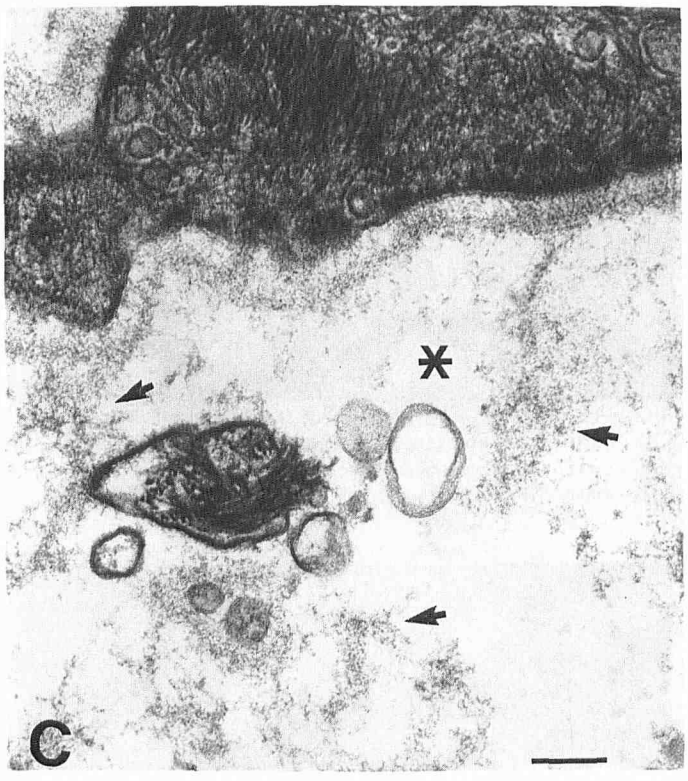


Figure 8. Electron micrographs of dermal-epidermal junctions of a non-blistered area of RDEB graft *(A)*, grafted healthy human skin *(B)*, and a blistered area of RDEB graft *(C)*. Both RDEB and healthy skin xenografts were biopsied 8 weeks after grafting. The *asterisk* marks the blister cavity. Both non-blistered *(A)* and blistered *(C)* areas of the RDEB xenograft significantly lacked well-formed anchoring fibrils (AF). Fragmented AF and interstitial collagen observed in the blister cavity reflect active collagenolysis (*arrows* in *C*). In contrast, the xenograft of healthy human skin *(B)* contained abundant, well-formed AF (*arrows* in *B*) comparable to that observed in ungrafted healthy skin. *Bar* in *A,B,C,* 0.18 μm.

Table II. Summary of RDEB Xenograft Characteristics

Examination	*Microscopic Findings*
LM	Subepidermal bullae
IF	W6/32 (+), 20.8.4s (−)
	BPA, laminin, type IV; (+) blister roof
	Type VII labeling lacking or absent
EM	Sub-lamina densa separation
	Rudimentary and/or diminished AF

after transplantation. In spite of the preservation of human vasculature in our grafts, the circulating blood cells and nutrients are most likely of murine origin. These circulating cells may have washed off with tissue fixation and staining procedures. We did not observe labeling of the human dermis in our xenografts with antibody against mouse class I antigen (20.8.4s).

We demonstrated here that, when grafted onto SCID mice, RDEB skin remains mostly human and retains the disease phenotype at least up to 24 weeks after transplantation. This RDEB xenograft model can serve as a complementary alternative to spontaneous animal models, and thus, could be used for in vivo experiments to explore the mechanisms of carcinogenesis and the potential basic biologic defects of this disease.

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